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The Development of Gene Therapy for the Treatment of Cancer

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Objective

The authors sought to develop new treatments for patients with cancer based on the genetic modification of immune lymphocytes and tumor cells designed to increase the host immune reaction against growing cancers.

Methods

Retroviral-mediated gene transduction was used to introduce genes into tumor-infiltrating lymphocytes (TIL), and these genetically altered TIL were administered to patients with cancer. Genes coding for cytokines were introduced into tumor cells, and these cells were used to immunize patients against their autologous cancers.

Results

In initial studies, the gene for neomycin phosphotransferase was introduced into the TIL of ten patients with advanced cancer to study the survival and distribution of TIL in humans. These studies showed that retroviral gene transduction is a safe and practical method for adding genes to human cells and led to clinical trials in which the gene for tumor necrosis factor (TNF) was inserted into TIL in an effort to increase their therapeutic effectiveness. Phase I trials are currently underway using TIL that secrete up to 100 times the normal level of TNF. More recently, animal experiments have revealed that transduction of tumor cells with cytokine genes can enhance tumor immunogenicity and, thus, increase the recognition of the tumor as foreign by the host. Clinical trials based on these observations have begun in which patients are immunized against their own autologous tumors that were transduced with the genes for TNF or interleukin-2.

Conclusions

Attempts at gene therapy for cancer are underway and have opened new possibilities for the development of cancer treatments.

The development of recombinant DNA technology and the rapid increase in understanding of the molecular basis of immunologic defenses against cancer have given rise to new opportunities for the development of cancer treatments. The ability to identify, isolate, and sequence functional genes and then transfer them from one cell to another provides unique opportunities for manipulating and amplifying biologic responses. The recent development of high-efficiency techniques for gene transduction using retroviruses has made the treatment of human diseases by gene transfer techniques a realistic possibility. This approach to treatment has been referred to as gene

therapy, which can be defined as a therapeutic technique in which a functioning gene is inserted into the cells of a patient to correct an inborn genetic error or to provide a new function to the cell.

Our prior studies were directed toward developing immunotherapies for the treatment of patients with cancer.[1,2] After extensive experimentation in animal models,[3,4] we developed treatment using interleukin-2 (IL-2) given either alone or in conjunction with lymphokine-activated killer (LAK) cells. These treatments were capable of causing objective regression of metastatic cancer in approximately 20% of patients with metastatic melanoma or metastatic renal cancer.[5-7] In 1986, we described the use of tumor-infiltrating lymphocytes (TIL) that could be grown from single-cell suspensions of murine tumors cultured in IL-2.[8,9] The adoptive transfer of TIL plus IL-2 was from 50- to 100-fold more effective than were LAK cells plus IL-2 in the treatment of established murine pulmonary metastases. These experimental studies led to pilot clinical trials in which patients with metastatic melanoma were treated with TIL and IL-2. Objective cancer regression occurred in approximately 35% of treated patients.[2,10] Studies in these patients have demonstrated that intravenously administered TIL traffic to and accumulate at tumor sites.[11,12]

In an attempt to improve on this immunotherapy and take advantage of the "homing" properties of TIL, we began efforts to modify TIL genetically to improve their therapeutic effectiveness.[13] We have now completed a clinical trial using TIL genetically modified with the bacterial gene coding for neomycin phosphotransferase[14] and have recently begun clinical studies using TIL transduced with the human gene coding for tumor necrosis factor (TNF) to treat patients with advanced cancer.[15] The results of these studies have led us to attempts to modify human tumors genetically for use in the immunization of patients with advanced cancer.[15] The development of these pilot trials for the gene therapy of cancer are briefly reviewed in this article.

METHODS

Growth of TIL

Procedures for the growth of human TIL have been published.[10,16] In brief, freshly resected tumor was minced into 3- to 5-mm^3 fragments and digested overnight in an enzyme medium consisting of collagenase type IV, hyaluronidase type V, and deoxyribonuclease type IV. The next day, the single-cell suspension was passed through a sterile wire mesh grid to remove undigested debris, the cells were washed three times, and viable cells were counted. If viability was less than 70% or the preparation was heavily contaminated with erythrocytes, the preparation was separated by Ficoll-Hypaque (Organon Telenika-Cappel, Durham, NC) gradient centrifugation to remove dead cells and erythrocytes and then washed again in saline solution.

The cell cultures were established by plating 5×10^5/mL of total cells of the single-cell suspension in culture medium composed of either serum-free AIM-5 medium (GIBCO, Grand Island, NY) or RPMI-1640 medium containing 10% human AB serum, with the additives, glutamine, amphotericin B, penicillin, streptomycin, 7200 IU/mL of IL-2, and 10% LAK cell supernatant. During the course of 2 weeks, the tumor cells gradually disappeared as the lymphocytes grew. When the lymphocyte number reached 1.5×10^6 cells/mL, these cells were split with fresh medium. When approximately 10^9 cells were reached, the cells were switched from tissue culture plates to gas-permeable cell culture bags and cultured until greater than 10^{11} TIL were obtained.

To administer TIL, they were first collected in saline using continuous-flow centrifugation and then filtered through a platelet administration set into a volume of 200 to 300 mL containing albumin and 450,000 IU of IL-2. The TIL were infused into patients over 30 to 60 minutes through a central venous catheter.

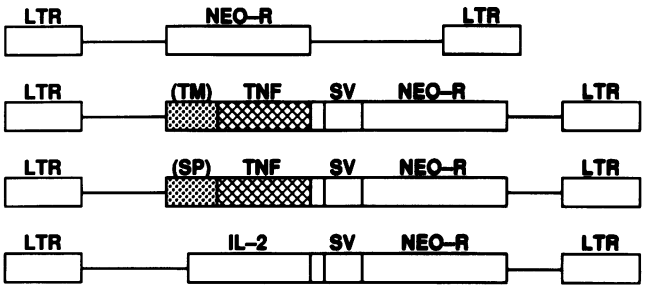

Figure 1. Retroviral vectors used to transduce TIL or tumor cells with the genes for neomycin phosphotransferase (NeoR), TNF, and IL-2. LTR indicates a long terminal repeat; SV, simian virus promoter; TM, transmembrane; and SP, signal peptide.

Production of Retroviral Vectors

Retroviral vectors were produced as previously described.[14,17-20] A diagram of the vectors used in this study is shown in Figure 1. The retroviral vector LNL6 containing the neomycin resistance gene was derived from the N2 vector, a derivative of the Moloney murine leuke-

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mia virus in which the *gag, pol,* and *env* genes were deleted and the bacterial NeoR gene, which encodes a bacterial enzyme, neomycin phosphotransferase, was inserted. This intracellular enzyme inactivates G418 (Gibco, Grand Island, NY), a neomycin analogue that is otherwise toxic to eukaryotic cells. LNL6 virions were produced by introducing LNL6 into the retroviral packaging cell line PA317. The bulk PA317 cell line was cloned, and the clone producing the highest viral titer was isolated. Retroviral vectors used in this study had titers between 2×10^5/mL and 3×10^6/mL colony-forming units when assayed for their ability to confer neomycin resistance to NIH-3T3 cells.

Retroviral vectors containing two genes were based on the LXSN backbone that has previously been described.[17-20] Either the TNF gene or the IL-2 gene was inserted under the transcriptional control of the retroviral LTR. The NeoR gene was under the transcriptional control of the simian virus 40 early-region promoter. These vectors are referred to as LTSN and LISN, respectively. In these vectors, an adenine–thymine-rich, 3′ untranslated region was excised before cloning the cytokine DNA into the retroviral backbone to avoid posttranscriptional control by this region.

To increase TNF secretion by transduced cells, a hypersecretor form of the TNF vector was prepared by replacing the 5′ transmembrane portion of the TNF complementary DNA by a gene sequence coding for the signal peptide from the γ-interferon gene.[21] This vector is referred to as LT-γsigSN.

The LNL6 vector and the LISN vectors were kindly supplied by Genetic Therapy Inc. (Gaithersburg, MD). The LTSN vectors were kindly supplied by the Chiron Corporation (Emeryville, CA) and Genetic Therapy Inc. The hypersecretor TNF vector was supplied by the Chiron Corporation.

Growth of Melanoma Tumor Cell Lines

Freshly resected melanoma specimens were minced into 3- to 5-mm^3 fragments and digested by the same enzyme mixture used to produce TIL. Tumor cell cultures were established in 24-well tissue culture plates in RPMI-1640 plus 10% heat-inactivated fetal bovine serum. Melanoma colonies were allowed to grow to confluence before passage to larger culture vessels. Melanoma tumor cells were identified using anti-GD3 monoclonal antibody (Mel-1, obtained from Signet Laboratories, Boston, MA).

Retroviral Transduction of TIL and Tumor

To transduce TIL retrovirally, 10^6/mL TIL were suspended in AIM-5 medium containing 6000 IU/mL of IL-2 in 24-well plates. An equal volume of the retroviral supernatant was added, containing protamine at a final concentration of 5 μg/mL (Eli Lilly, Indianapolis, IN). After 24 hours, 1 mL was aspirated from each well and replaced with 1 mL of freshly thawed retroviral supernatant. The medium was supplemented with fresh IL-2 and protamine. Two to 3 days later, TIL were subcultured into medium containing either 0.3 or 0.5 mg/mL of G418 for 5 days before being returned to medium without G418.

The melanoma tumor lines were transduced with the retroviral vectors in a manner similar to that of TIL but in the absence of IL-2. Selection of transduced cells was performed by continuous growth of cultures in 0.4 mg/mL of G418 beginning 24 hours after transduction. In contrast to TIL, tumor cells were grown continuously in G418.

Clinical Protocol

All patients treated in these clinical trials had metastatic melanoma that had not responded to standard effective therapy. The patients received up to 3×10^{11} TIL administered in one to four infusions of up to 300 mL each. Approximately 2 hours after completing the TIL infusions, the patients began to receive intravenous administration of recombinant IL-2 every 8 hours at varying doses depending on the protocol.

Patients receiving NeoR-transduced TIL received a single infusion in conjunction with nontransduced TIL.[14] These patients received a second cycle of TIL approximately 2 weeks after completing the first cycle.

Those receiving TIL modified by the TNF gene received escalating doses of transduced TIL alone, starting at 10^8 TIL and escalating every 3 to 7 days to a final level of 3×10^{11} TIL. Early patients received no IL-2, but in subsequent ones, permission was obtained from the Food and Drug Administration to administer IL-2 at a dose of 180,000 IU/kg every 8 hours after TIL administration.

Patients receiving tumor cell immunizations received 1 to 2×10^8 transduced tumor cells, which were injected subcutaneously into the anterior thigh. Approximately 2 cm distally, two intradermal injections of 1 to 2×10^7 transduced tumor cells were administered. Tumor immunizations were performed as an outpatient procedure.

All clinical protocols involving gene transfer were approved by the Investigational Review Board of the National Cancer Institute, the National Institutes of Health Biosafety Committee, the Recombinant DNA Advisory Committee, and the Food and Drug Administration.

Table 1. GENE THERAPY WITH TIL: NeoR TRANSDUCED TIL—PATIENT CHARACTERISTICS

Age/Sex	Prior Treatment	Sites of Evaluable Disease	No. of Transduced Cells Infused ($\times 10^{-10}$)
52/M	Wide local excision	Lung, liver, spleen	7.1
46/F	Amputation finger, lymph node dissection	Lymph nodes, intramuscular	13.2
42/M	Wide local excision, lymph node dissection, melanoma "vaccine," interleukin-2/alpha-interferon	Lung, subcutaneous	20.0
41/M	Wide local excision, lymph node dissection, interleukin-2/alpha-interferon	Lung, liver, lymph nodes, subcutaneous, brain	3.3
26/F	Wide local excision	Lung, lymph nodes, subcutaneous	6.2
38/M	Wide local excision, lymph node dissection, chemotherapy, BCG "vaccine," interleukin-2	Lymph nodes, subcutaneous	10.0
30/M	Wide local excision, excision of recurrence, interleukin-2, alpha-interferon	Lymph nodes, intraperitoneal	2.9
43/M	Wide local excision, excision of recurrence, interleukin-2, alpha-interferon	Brain, subcutaneous	7.7
30/M	Wide local excision, lymph node dissection, chemotherapy, interleukin-2	Subcutaneous	15.6
53/M	Wide local excision, lymph node dissection, melanoma "vaccine"	Lymph nodes, subcutaneous	3.0

RESULTS

Gene Modification of TIL to Confer Neomycin Resistance (NeoR)

Our first effort to modify human TIL genetically involved introducing the gene for a bacterial enzyme coding for neomycin phosphotransferase, which transmits resistance to the antibiotic G418.[14]

Ten patients with advanced melanoma were treated in this clinical protocol. The characteristics of these patients are shown in Table 1. An example of the growth of transduced and nontransduced cultures is shown in Figure 2. Multiple safety tests were performed on the viral supernatant, transduced TIL, and sera from the patients as detailed in Table 2. All safety tests that were performed on all patients yielded negative results. There was no evidence, in any test, that replication-competent virus was present either in the viral supernatants or in the TIL administered to the patients. Furthermore, sensitive tests to detect the presence of antibody to viral envelope protein in the sera of patients at varying times after cell administration also had negative findings.

Using the polymerase chain reaction technique, transduced cells could be readily identified in the peripheral blood of patients for the first 21 days after cell administration. The cells could only occasionally be identified in the peripheral blood at later times, although they were found in one patient at 189 days. Gene-modified cells could also be identified in tumor biopsy specimens up to 64 days, indicating that at least some of the modified cells traveled to the tumor deposits.

These patients had been extensively pretreated before entering this gene-modified TIL protocol. All patients had undergone surgical excision of their primary melanoma, and six of the patients previously had not responded to immunotherapeutic regimens based on high doses of IL-2. Two patients in this protocol had an objective cancer response. One had a partial response lasting 2 months, and the other sustained a complete regression of metastatic cancer to the lungs, subcutaneous tissue, soft palate, and both tonsils that lasted 38 months.

This clinical trial demonstrated that retroviral-mediated gene transduction could be safely used to modify human cells administered to patients. This trial led us to subsequent efforts to introduce genes that were intended to improve the therapeutic potency of TIL.

Gene Therapy for Cancer Using TIL Transduced with the Gene for TNF

Studies of therapy with TIL in experimental animal tumor models had shown that cytokine secretion, especially of TNF and γ-interferon, was important in the *in vivo* therapeutic impact of TIL against established lung metastases.[22] We selected the TNF gene for genetic modification of TIL because of the effectiveness of TNF in

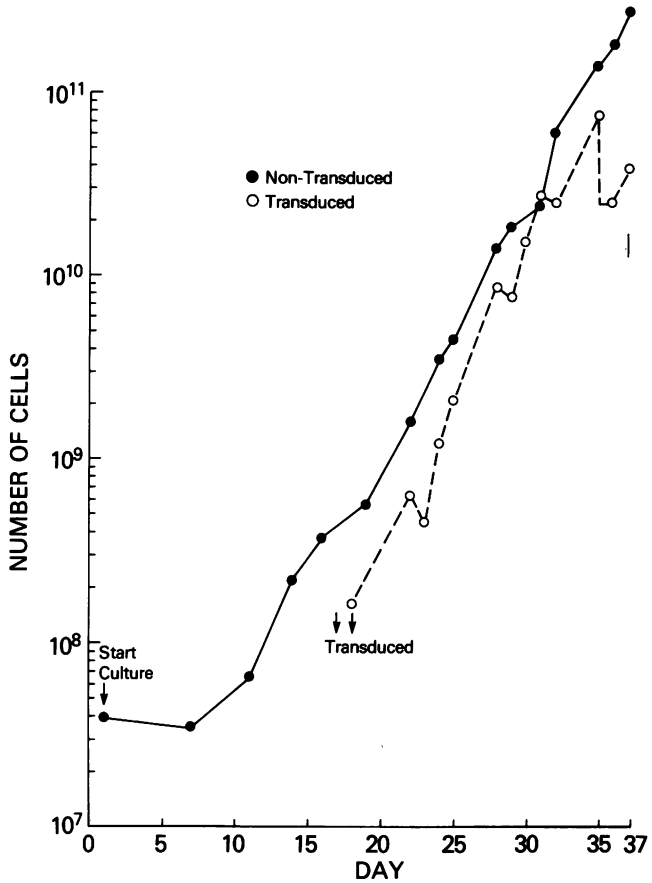

Figure 2. Example of the growth pattern of TIL (solid line) and TIL transduced with the NeoR gene (dashed line).

murine tumor models.[23,24] A single injection of TNF can cause coagulative necrosis of large murine tumors within hours after injection. The toxicity of TNF when injected systemically in humans, however, has prevented the administration of the doses of TNF that mouse models predict are required to see therapeutic effects in humans. Because TIL accumulate in tumor deposits, we hypothesized that TIL, transduced with the gene for TNF and producing large amounts of this cytokine, would enable the delivery of sufficient quantities of TNF to the tumor to result in its destruction without the systemic toxicity seen when large amounts of TNF were injected intravenously. We thus used retroviral vectors capable of inserting two genes into TIL (Fig. 1). Because TNF can be associated with significant hemodynamic toxicity, a cautious escalation of the number of gene-modified cells was performed first in the absence of IL-2. Only later did we add IL-2 to the treatment regimen. An outline of the treatment protocol approved by the Food and Drug Administration is shown in Table 3. Three early patients were treated with unselected cells, but the subsequent seven patients received cells selected in G418 to increase the fraction of transduced cells present in our TIL cultures. Unselected cultures had a mean (± SEM) per cent of transduced cells of 3.2 ± 1%. In a series of transduced TIL cultures, cells selected for 5 days in either 0.1, 0.3, or 0.5 mg/mL of G418 had 9.0 ± 2.0%, 40.9 ± 6.5%, and 57.8 ± 7.5% transduced cells, respectively. Examples of the production of TNF by nontransduced and transduced selected human TIL at 25 to 35 days after transduction is shown in Table 4.[25] We have recently begun using a hypersecretor TNF vector in which the transmembrane domain of the TNF molecule has been re-

Table 2. SAFETY TESTS PERFORMED IN PROTOCOLS USING RETROVIRAL VECTORS FOR THE GENE THERAPY OF CANCER

Viral supernatant
Viral titer (on 3T3 cells)
Sterility
Mycoplasma
Injection into mice
Viral testing
MAP test
3T3 amplification S+L− assay (ecotropic, xenotropic, and amphotropic)
Electron microscopy
Transduced TIL
3T3 amplification S+L− assay
Reverse transcriptase assay
Endotoxin assay
IL-2 withdrawal
G418 selection
Southern blot or PCR to detect inserted gene
NPT assay
Cytotoxic profile
Phenotypic profile
Patient
Test cells from patient for virus (3T3 amplification)
Serum test for antibody to helper virus antigen 4070A (Western Blot)

Table 3. PROTOCOL SCHEMA—TNF GENE MODIFIED TIL

1. Escalate TNF-TIL twice weekly; no IL-2.
   10^8 cells
   3×10^8
   10^9
   3×10^9
   10^{10}
   3×10^{10}
   10^{11}
   3×10^{11}
2. Reduce cell dose to one-tenth maximum tolerated dose. Escalate as in (1) every 3 weeks with 180,000 IU/kg of IL-2.
3. After three patients, discuss with the FDA. (Possibly start at a higher cell dose with IL-2.)

Table 4. SECRETION OF TNF BY TIL TRANSDUCED WITH THE TNF GENE

Patient	Nontransduced	TNF Vector	Hypersecretor TNF Vector
	TNF (pg/10^6 cells/24 hours)		
1	5	380	2806
2	104	104	657
3	40	185	983

placed by the signal sequence of the γ-interferon molecule.[21] This modification leads to increased secretion of TNF. Of the ten patients who have received TIL modified by the TNF gene, six previously had not responded to TIL therapy with nontransduced TIL. One of these patients, the only patient who thus far has responded, showed an objective response that is now ongoing 2 years after treatment with TNF-TIL. Dose escalation of the number of transduced TIL and the dose of IL-2 are continuing in this trial.

Gene Therapy for Cancer Using Tumor Cells Transduced with the Genes for TNF or IL-2

Various studies in experimental animal models have shown that the introduction of cytokine genes into tumor cells can result in decreased tumorigenicity and an increased production of specific cytolytic antitumor immune cells by the host.[26] After extensive animal experimentation, we initiated a protocol in which human tumor cells were grown in culture and transduced with the gene for either TNF or IL-2[27] before being used to immunize the autologous patient. An outline of this gene protocol is shown in Table 5. In animal models, the injected tumors that were transduced with either of these genes grow and then spontaneously regressed at the injection site. We have thus far treated three patients with autologous tumor cells modified with the gene for TNF and two with the gene for IL-2. Similar to what occurred in animal models, none of the tumors grew at the injection site in the anterior thigh, and there was no evidence of viable tumor cells when these sites were surgically resected approximately 3 weeks after injection.

A second important aspect of this protocol is now under study. At the time of resection of this tumor, draining inguinal lymph nodes are resected, and techniques are being developed to grow these cells in culture for subsequent administration to the patient. Thus, in this protocol, patients undergo both an immunization and an adoptive cell transfer in an attempt to mediate tumor regression. Currently, one patient has had a partial regression lasting 4 months. This protocol is currently ongoing but has already demonstrated that patients can be safely immunized with their own autologous, gene-modified tumor cells, which are rejected after subcutaneous administration.

DISCUSSION

During the last decade, effective biologic therapies for selected patients with advanced cancer have been developed based on altering natural host immune defenses against the malignancy.[28,29] These efforts have been stimulated by the development of recombinant DNA technology, which has made biologic reagents, previously available only in minute quantities, available in amounts large enough for use in humans. Recombinant IL-2 has no direct impact on cancer cells but can mediate significant antitumor activity through modification of host immune defenses. After extensive animal experimentation and human clinical trials, IL-2 was accepted by the Food and Drug Administration in 1992 as the only approved treatment for use in patients with metastatic renal cell cancer.

The predominant role of the cellular immune system in immunologic reactions against foreign tissue stimulated research to identify immune cells capable of recognizing antigens uniquely present on human cancers. The description of LAK cells in 1980 provided a means for generating large numbers of cells in both experimental animals and in humans that were capable of distinguishing fresh cancer cells from fresh normal cells.[30,31] In experimental animal models[3,4] and later in humans,[5–7] the adoptive transfer of LAK cells plus IL-2 was shown to be able to mediate antitumor effects in selected patients with renal cell cancer, melanoma, and to a lesser extent, colon cancer and non-Hodgkin's lymphomas.

A search for more potent cells to use in adoptive immunotherapy led to the description of TIL, cells that recognize unique antigens in murine and human tumors

Table 5. PROTOCOL SCHEMA FOR CYTOKINE GENE MODIFIED TUMOR

1. Tumor resected as part of standard treatment.
2. Tissue culture line established.
3. Cytokine gene transduced into culture and cultures selected in G418 (cytokine greater than 100 pg/10^6 cells/24 hours).
4. Inject 2×10^8 gene-modified tumor subcutaneously into right thigh and 2×10^7 cells intradermally at two nearby sites.
5. Three weeks later remove draining lymph node and grow *in vitro* in IL-2.
6. Adoptive immunotherapy using these cells plus IL-2.

in a major histocompatibility complex-restricted fashion.[8,9,32] The adoptive transfer of TIL was from 50- to 100-fold more potent than that of LAK cells in animal therapy models. In the initial clinical trials with TIL, objective response rates of 35% to 40% occurred in patients with malignant melanoma. The response rates to TIL were almost twice those obtained with IL-2 alone or LAK cells plus IL-2.[2,10] Studies with indium-111-labeled TIL injected intravenously into patients with cancer demonstrated that these cells traveled to and accumulated in tumor deposits.[11,12] These studies suggested that TIL might be used as vehicles for delivering molecules to tumor sites that could aid in the antitumor effectiveness of TIL. It was with this goal in mind that we began studies of the genetic modification of TIL.[13,14]

Although many methods exist for introducing foreign genes into cells, the only method with sufficient efficiency for practical use in human trials involved the use of genetically engineered retroviruses. We used a genetically modified murine Moloney leukemia virus containing the gene of interest but engineered so that its viral coding sequences were removed. Because a major purpose of this first study was to evaluate the safety of these techniques for human gene therapy, we introduced a gene that did not transmit toxicity to the TIL. We selected the gene for neomycin phosphotransferase, an enzyme that conferred resistance to the antibiotic G418. This enzyme is not naturally present in human cells, and thus, the only cells capable of surviving in G418 are cells transduced with this gene. These studies were designed to determine the distribution and survival of intravenously injected gene-modified TIL in the body. The polymerase chain reaction technique, used to identify transduced cells could detect one transduced cell in 100,000 nontransduced cells. Alternatively, transduced cells could be identified by growing cells or tissues isolated from the patient in G418.

Because this clinical trial was the first to introduce foreign genes into humans, considerable attention was paid to the potential dangers of using retroviruses for gene modification in humans.[14] A large number of safety tests were performed to ensure that this procedure was safe for the patient and for health care personnel (Table 2). Using retroviruses, foreign genes are randomly integrated into the chromosomes of the host cell. There was concern that changes might be induced in the lymphocytes by the introduction of the foreign genes that would either inactivate vital cellular genes or result in malignant transformation of the transduced cells. Because the foreign genes used in this protocol were transduced into cells in culture, we performed extensive tests on the cells to show that their phenotype and functional properties were not altered by the gene transduction. We also demonstrated that the transduced TIL still required IL-2 for growth in culture before administering the cells to patients.

Although these retroviruses could not replicate and were not pathogenic, there was concern that they might recombine with endogenous viruses to cause the production of replication-competent pathogenic viruses. Sensitive tests, using amplification on permissive NIH-3T3 cell lines with subsequent testing for replication viruses by S+L− assays, were used to check all viral and TIL preparations. In addition, the gene sequences of the packaging cell lines and the vectors were modified to reduce the possibility that any recombination could occur that would generate replication-competent virus.

These initial studies were designed to determine the long-term traffic patterns and distribution of TIL in the body. Indium-111 has a half-life of only 2.9 days and, thus, could not be used for long-term studies of TIL survival *in vivo*. We were able to find circulating transduced cells in peripheral blood up to 189 days and accumulations of TIL in tumor deposits up to 64 days after lymphocyte infusion.

These initial studies helped establish the safety of using retroviruses to modify human lymphocytes, and they stimulated studies in two directions. Led by Dr. Michael Blaese and Dr. French Anderson, the gene for adenosine deaminase was transferred into the lymphocytes of children with severe combined immunodeficiency disease resulting from this enzyme deficiency. The efforts described in this review were devoted to "designing" lymphocytes with genetic modifications that could improve their efficacy for treating cancer.

The gene for TNF was chosen for introduction into TIL because of the remarkable antitumor activity of this cytokine in murine models. Studies in experimental animals suggested that approximately 400 μg/kg of TNF was required to induce anticancer effects. Humans, however, can tolerate only 8 μg/kg/day. We hypothesized that TIL transduced with the gene for TNF and, thus, producing large amounts of TNF would accumulate at tumor deposits and produce the high local concentrations required to mediate tumor toxicity without exposing the patient to high systemic doses of TNF.

We used vectors that could insert two genes instead of one, the TNF gene and the NeoR gene. A variety of problems were encountered in obtaining high production of the products of both genes, possibly because of the promoter inhibition effects that occur when two genes are transduced with a single retrovirus. We are currently attempting to develop improved vectors that can lead to increased TNF production. Recently, we have begun using a modified vector that utilizes a gene for TNF, which was altered by replacing its transmembrane domain with the gene segment coding for the γ-interferon signal peptide.[21] This modified TNF is not incor-

porated into the membrane of the cell and, thus, results in the secretion of larger amounts of TNF. We are currently in the midst of a phase I escalation using transduced TIL secreting the altered TNF. We are using increasing numbers of cells first without concomitant IL-2 and then adding low doses of IL-2. As this protocol continues, increasing numbers of cells will be given along with higher doses of IL-2.

Various genetic modifications, in addition to the introduction of the TNF gene, are being investigated. Studies are being conducted in which genes coding for γ-interferon are inserted into TIL because of the ability of this cytokine to increase major histocompatibility complex and tumor antigen expression on tumor cells. The introduction of genes coding for chimeric T-cell receptors into TIL could enhance their ability to recognize tumor antigens. Other studies are directed at inserting the gene for the IL-2 receptor to make TIL more sensitive to IL-2 and, thus, allow the administration of lower doses of IL-2 with consequent decreased toxicity.

More recently, efforts have begun to modify genetically tumors to increase their immune recognition. In animal models, introducing a variety of cytokine genes into tumors can increase immunogenicity and decrease tumorigenicity.[27] In our own studies, we have shown that introducing the genes for TNF, IL-2, and γ-interferon can all increase immune recognition of murine tumor cells[33-35] and, in some cases, give rise to lymphocytes that can be used for the successful adoptive immunotherapy of established metastases. Based on these animal studies, we have begun clinical trials in which patients with advanced cancer are immunized with autologous tumor cells that secrete large amounts of either TNF or IL-2. In these initial studies, we have injected more than 10^8 live nonirradiated tumor cells secreting either TNF or IL-2. In each of the first five patients, a local area of erythema and induration developed that lasted for about 2 weeks. When the injection site was excised 3 weeks later, no viable tumor could be found. We are currently attempting to isolate lymphocytes from these injection sites and draining lymph nodes that might be used for adoptive therapy in these patients with advanced disease.

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Discussion

DR. MURRAY F. BRENNAN (New York, New York): I rise not because I know anything about the subject, but only to say that Dr. Rosenberg asked me if nobody commented on his paper, would I get the discussion started. I did not expect to be asked! I spent a wonderful 6 years working with Dr. Rosenberg, during which time he tried very hard to explain some of the intricacies of immunology when it was still an understandable science. I would like the audience to know that he failed miserably. The moment he got beyond interleukin-2 or -3, I was lost.

What I would like to ask is if you would conjecture a little further about other methods of genetically modifying cells in a cancer patient. You mentioned briefly modified surface antigens and modified infiltrating lymphocytes. I think if the audience would allow it, his speculation on that would be much more exciting than any comment that I might make.

Finally, I hope others understand that I stood up to say, Steve, most of us admire greatly the efforts that you, as a surgeon, have made to improve survival in the patient with unresectable cancer.

DR. ALLAN D. CALLOW (St. Louis, Missouri): Dr. Rosenberg, your presentation was splendid, of course, as you must have been told many times. I have a question. Control of expression of an inserted foreign gene is extremely important. The prolonged production of tumor necrosis factor (TNF) after therapeutic need has passed can be detrimental. Retroviral promoters are known to lose effectiveness over time *in vivo*. Is this the mechanism by which TNF expression falls off in your method? Is it due to withholding interleukin-2 thereby reducing tumor infiltrating lymphocytes replication, or could it be both? Thank you again for a splendid talk.

DR. WANEBO (Providence): Steve, I guess all of us continue to be impressed by the enormous amount of work that you continue to pour out.

I have three questions. You've shown that the neomycin transfected cells can last over 180 days, and my question is, with these tumor necrosis factor (TNF)-producing cells, how long do they last? Maybe you can at least conjecture on this. Are these immortalized cells or are they finally eliminated?

Second, I've always been intrigued with the idea that you are able to induce the lymphocyte to make TNF. I guess I was under the impression, maybe incorrectly so, that this was a macrophage-generated lymphokine, but it's intriguing that you can induce lymphocytes to do this. Are these cells totally potential? Can we get them to make anything we want?

Third, your vaccination with the tumor cells transfected with interleukin-2 (IL2) or TNF definitely induces a regression. The question is: What is going on here? Is this truly an immunologic event mediated through T cell recognition and then generation of cytotoxic lymphocytes, or is this really just a localized LAK cell effect due to the fact that these lymphocytes are producing huge amounts of IL2 in a local area?

DR. ALI NAJI (Philadelphia, Pennsylvania): Dr. Rosenberg, I also rise to congratulate you on this outstanding work. I have three specific questions regarding the biology of the tumor infiltrating lymphocyte (TIL) cells and with respect to some of the differentiation antigens.

Initially, one of the issues with TIL was the percentages of cells that ultimately home to the tumor deposits. Does transfection and genetic engineering of tumor necrosis factor (TNF) modify the homing pattern to the tumor?

The second question is with respect to the work being done by Lindsay' group or the role of the B-7 CD-28. Would you tell us if your TIL cells do express CD-28 and the role of B-7, particularly the reason for it with the B-7 engineering in the melanoma cells and regression of the tumor?

The third question is with respect to another determinant that a German group has been working with, the CD-44, which appears to be important in metastatic potential of the tumor cells. Could you elaborate on the potential of TIL cells and CD-44?